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The biosynthesis of cholesterol in the developing chick embryo*

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SUMMARY

Mevalonic acid-2-C^{14} was injected into the yolk of incubating fertile hens' eggs on the sixth day of incubation. A nonsaponifiable fraction (NSF) containing labeled components was isolated from the yolk sac and from the embryo throughout the remainder of the incubation. The specific activity of both the yolk sac and embryonic NSF increased during the first 3 days following injection of the labeled precursor and then decreased from the remainder of the incubation. The total activity of the yolk sac and embryonic NSF increased during the incubation. The largest increment in total radioactivity of the yolk sac NSF occurred after the 12th day of incubation and that of the embryonic NSF after the 15th day. A high level of labeled squalene was found in the yolk sac NSF. Labeled cholesterol was found in both the yolk sac and embryo. About 3% of the recovered activity of the NSF was found in the brain tissue of chicks hatched from injected eggs.

Rittenberg and Schoenheimer (1) concluded that there is no synthesis of cholesterol in the incubating egg since an incorporation of deuterium from deuterium oxide was not observed. Bernhard (2), however, was able to demonstrate an incorporation of deuterium into embryonic cholesterol. More recently, Stokes *et al.* (3) found that acetate-C^{14}, injected into the allantoic cavity subsequent to the tenth day of incubation, labels cholesterol in the embryo and chorioallantoic membranes in the last half of the incubation. Tsuji *et al.* (4) after studying the levels of free and esterified cholesterol in incubating eggs, concluded that there is no net cholesterol synthesis but that yolk cholesterol is esterified and transferred to the embryo. This transfer is first observed about the tenth day of incubation and is especially active 2 days prior to hatching.

The present work was undertaken to determine whether or not mevalonic acid-2-C^{14} (MVA-2-C^{14}), a precursor of sterols (5), furnishes labeled components of the nonsaponifiable fraction (NSF) in the developing chick embryo. Concurrently, a study of the levels of radioactive NSF in the embryo and yolk sac throughout incubation was carried out and the presence of labeled cholesterol and squalene (6) was established.

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† The data in this work are taken from a thesis presented to the Faculty of the Graduate School of Cornell University in partial fulfilment of the requirements for the degree of Doctor of Philosophy. Present address: Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire.

METHODS

Injection of DL-*MVA-2-*C^{14} *into Incubating Eggs and Isolation of the NSF.* Eggs from White Leghorn hens were incubated at 99 ± 1° F under proper conditions of humidity. On the sixth day of incubation, 0.5 ml of a sterile, aqueous solution of 0.0276 mg of DL-MVA-2-C^{14}, with a specific activity of 1.1 mc/mmole, was injected into the yolk of each egg. The injection was made with a tuberculin syringe equipped with a No. 22 needle, $1^1/_4$ in. long. In the first experiment, duplicate eggs were harvested at 5 min and at 8, 12, 18, and 24 hr after injection and then daily for the remainder of the incubation. In a later investigation, pooled samples were taken on the tenth day of incubation or on the fourth day after injection of the MVA-2-C^{14}. Eggs containing viable embryos were divided into three main fractions: washed embryo, washed yolk sac drained of yolk and combined with washed extra-embryonic membranes, and the yolk itself.

The NSF was isolated in the usual way (7). Saponification with 2 N alcoholic KOH was performed routinely overnight in 250-ml Erlenmeyer flasks at steam bath temperature. When intermediates were to be isolated, the saponification was carried out by refluxing for 3 hr

under nitrogen. The cooled hydrolysate was extracted five times with equal volumes of 30–60° petroleum ether. The extract was filtered and the solvent removed at steam bath temperature for daily, small isolations or by the use of a flash evaporator at 45° for batch procedures. The NSF for the isolation of intermediates was stored in the dark under nitrogen.

Radioassay was performed either with a gas flow counter equipped with a thin window or with a Packard Tri-Carb liquid scintillation spectrometer.

Chromatography of the Isolated NSF. The NSF obtained from pooled embryos and from pooled yolk sac-membrane fractions isolated on the tenth day of incubation was fractionated in each instance by alumina column chromatography using 150 g alumina, activity II (8), packed to a height of 22 cm in a glass column 3.0 cm in diam. The elution rate was held at 7–12 drops/10 sec. The fractions were collected by using a Gilson Medical Electronics Model V15[2] fraction collector adjusted to deliver a volume of 5.0 ml to each tube. Immediately after delivery into the test tubes, aliquots were taken for radioassay by liquid scintillation counting. Appropriate aliquots were removed for additional study of the composition of the peaks obtained. The solvent changes are given in Table 1.

RESULTS

Incorporation of MVA-2-C^{14} into the Embryo and Yolk Sac-Extra-Embryonic Membranes Throughout Incubation. The specific activity in cpm/mg was determined by gas flow counting of the dried and weighed NSF isolated from the embryo and from the yolk sac-membrane fractions of two eggs harvested daily. The average of the specific activities of the duplicate samples *versus* the time after injection or the day of incubation is shown in Fig. 1. The specific activity of the NSF from the yolk sac-membrane fraction increased rapidly until the third day after injection when a plateau of 80 cpm/mg was reached. This plateau was maintained until the 15th day of incubation after which the specific activity of the NSF of the yolk sac-membrane fraction decreased. The specific activity of the NSF isolated from the embryo also reached a peak on the third day after injection of the labeled substrate and then decreased during the remainder of the incubation.

TABLE 1. SOLVENTS USED IN THE CHROMATOGRAPHY OF THE NONSAPONIFIABLE FRACTION ISOLATED ON THE TENTH DAY OF INCUBATION FROM POOLED YOLK SAC-MEMBRANE AND POOLED EMBRYO SOURCES

Solvent	Tube No. at Solvent Changes†	
	Yolk Sac-Membrane	Embryo
Petroleum ether*	1	1
NSF in petroleum ether	19	16
Petroleum ether	32	28
10% benzene in petroleum ether	58	53
100% benzene	101	95
20% diethyl ether in benzene	169	160
100% diethyl ether	260	250
20% chloroform in diethyl ether	304	293
100% chloroform	351	335
95% ethanol	533	518

* The petroleum ether used was washed with concentrated sulfuric acid and distilled at 36–38.5°.

† The conditions employed in the column chromatography are given in the text.

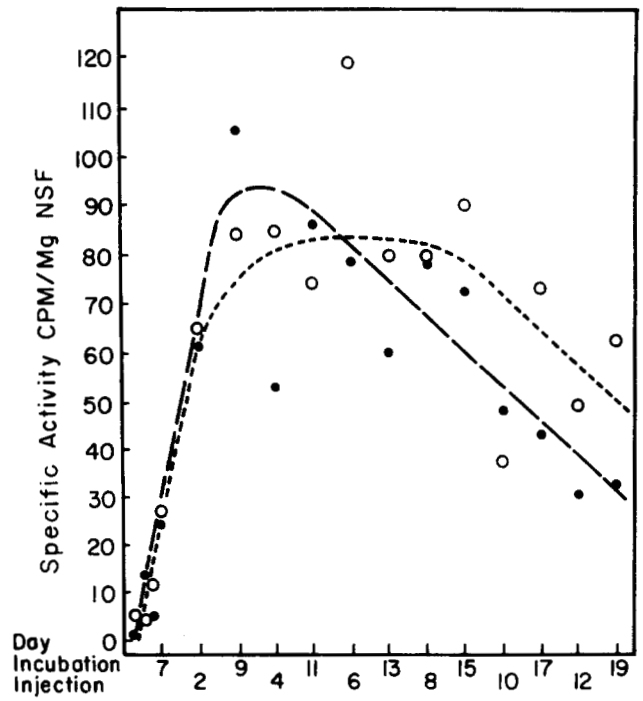

FIG. 1. The specific activity (cpm/mg) of NSF vs. the day of incubation and/or the time after injection of DL-mevalonic acid-2-C^{14} on the sixth day of incubation. Embryo —— ● ——, yolk sac-membranes - - - ○ - - -.

The total activity determined by liquid scintillation counting of the NSF from the embryo and from the yolk sac-membrane fraction is illustrated in Fig. 2. The total activity observed in the embryo and in the yolk sac-membrane fraction during the first 48 hr following injection of the labeled substrate is given in Table 2.

A labeled NSF was observed in the yolk sac-membrane fraction as early as 5 min after injection of the MVA-2-C^{14} and in the embryonic NSF within 8 hr after injection. The total activity of the NSF from the

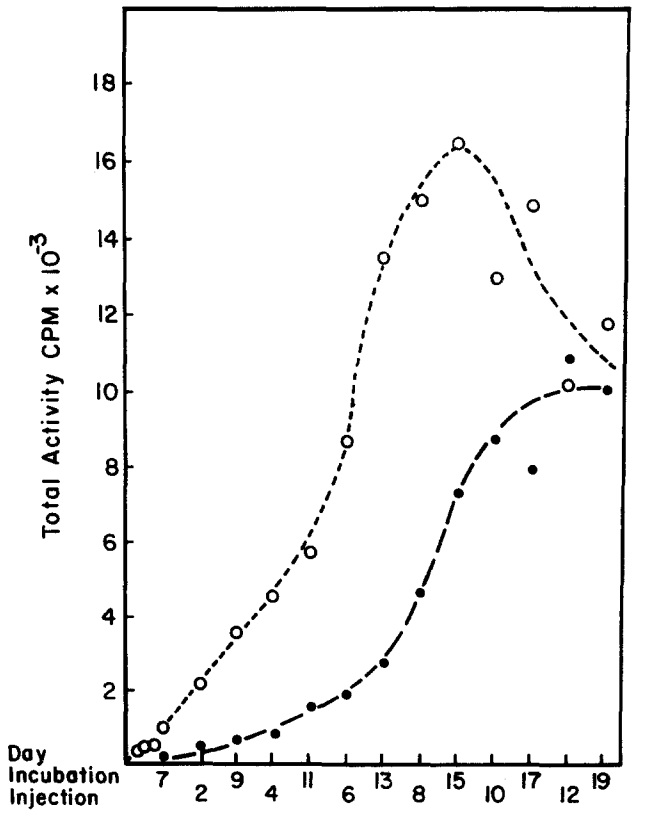

FIG. 2. The total activity (cpm) for the nonsaponifiable fraction of the embryo — — ● — — and of the yolk sac-membranes - - - O - - - vs. the day of incubation and/or the time after injection of DL-mevalonic acid-2-C^{14} on the sixth day of incubation.

yolk sac-membrane increased linearly until the 12th day of incubation when a more rapid increase occurred up to the 15th day of incubation. The total activity in this fraction then began to decrease. The total activity of the embryonic NSF increased almost linearly until the 14th day of incubation at which time it exhibited a rapid increase up to the 18th day of incubation.

Another phase of sterol syntheses may occur in the yolk sac beginning on the 12th day of incubation since a rapid increase in the total activity was observed in the NSF of this component. This is further supported by the observation that the total activity in the embryo rapidly increased shortly after the increase was observed in the yolk sac. This latter synthesis of a NSF may be coupled with an increased transfer of unlabeled sterol from the yolk since the specific activity of both the yolk sac and embryo decreased during this period.

Distribution of Radioactive NSF in the Developing Embryo and in the Hatched Chick. An egg, which had been injected on the sixth day of incubation with 87,000 cpm (gas flow counting) of MVA-2-C^{14}, was separated on the 19th day of incubation, giving an embryo from which the liver was dissected. The NSF of the separated parts was isolated and radioassayed with a gas flow counter, giving the following specific activities in cpm/mg: liver, 40; embryo, 12; yolk sac, 37; yolk, 3; and extra-embryonic membranes, 15.

TABLE 2. AVERAGE TOTAL RADIOACTIVITY FOUND IN THE EMBRYO AND IN THE YOLK SAC-MEMBRANE NONSAPONIFIABLE FRACTION SHORTLY AFTER THE INJECTION OF DL-MEVALONIC ACID-2-C^{14}

Time After Injection	Total Radioactivity	
	Embryo	Yolk Sac-Membrane
	cpm	*cpm*
5 min	1	12
8 hr	43	366
12 hr	102	421
18 hr	44	500
24 hr	115	1035
48 hr	463	2216

A group of six chicks, which had hatched 7 days previously from eggs injected on the sixth day of incubation, was sacrificed; several tissues were pooled; and the NSF was isolated and radioassayed, giving the following specific activities in cpm/mg: liver, 49; absorbed yolk sac plus yolk, 50; and brain, 13. The total activity in cpm calculated per chick was: liver, 1,044; absorbed yolk sac plus yolk, 1,227; brain, 102; and carcass minus crop and gizzard, 1,344. Therefore, the percentage of recoverable activity found in the NSF from these tissues was: liver, 28%; absorbed yolk sac plus yolk, 33%; brain, 3%; and carcass, 36%.

Separation of a Labeled NSF. Another series of eggs was injected on the sixth day of incubation with 33,000 cpm (liquid scintillation counting) of DL-MVA-2-C^{14} per egg. On the tenth day of incubation, 20 eggs were separated into pooled embryo and pooled yolk sac-membrane fractions. The NSF of each fraction was isolated as described in the section on Methods and radioassayed by using liquid scintillation counting. The total activity isolated from the embryos was 42,000 cpm while that from the yolk-sac-membrane was 116,000 cpm. The percentage of total recovered activity would be 24% of the injected activity. By the Liebermann-Burchardt reaction, the amount of sterol isolated was determined to be 87 mg from the embryos and 193 mg from the yolk sac-membrane. The specific activities were 446 and 604 cpm/mg of the Liebermann-Burchardt positive material from the embryo and yolk sac-membrane, respectively.

The NSF from the pooled embryos and from the pooled yolk sac-membrane fractions was separated by

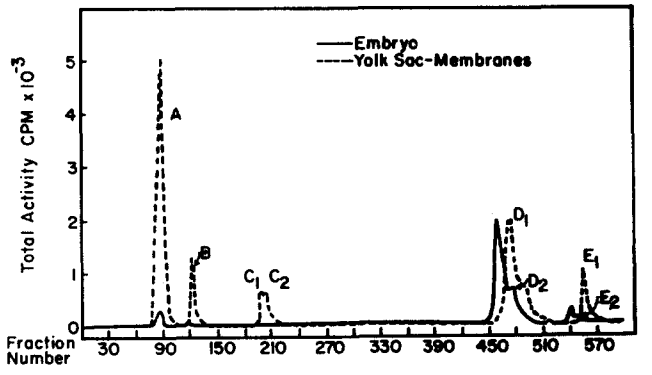

FIG. 3. Chromatographic pattern for the elution from an alumina column of the nonsaponifiable fraction obtained from the pooled embryo and from the pooled yolk sac-membrane harvested on the tenth day of incubation or fourth day after the injection of DL-mevalonic acid-2-C^{14}. The conditions are given in the text and in Table 1.

the techniques of column chromatography described in the section on Methods. The chromatographic pattern is given in Fig. 3. The total activity found in each peak, the percentage of recovered activity for each peak, and the specific activity in cpm/mg of Liebermann-Burchardt positive sterol are reported in Table 3.

It can be seen that the radioactivity in the "squalene peak," A, in the yolk sac-membrane fraction is over ten times that found in the embryo. The "sterol peaks," D, in the two fractions appear to be equal; however, the total activity in the yolk sac-membrane peak is slightly greater than that found in the embryo peak(s). At the same time, the total activity in the "sterol peak" of the yolk sac-membrane is almost equal to that of the "squalene peak" of the same yolk sac-membrane. The colorless oil from the central fraction of yolk sac-membrane A peak and the recrystallized material from the central region of the embryo-D_1 peak exhibited IR spectra similar to those given by squalene and cholesterol, respectively, analyzed on the same instrument.

DISCUSSION

Although there has been uncertainty as to whether or not cholesterol biosynthesis occurs during the development of the chick embryo (1, 2, 4), the incorporation of acetate-C^{14} into cholesterol and desmosterol has been demonstrated during the latter half of the incubation (9). The present work demonstrates that MVA-2-C^{14} is incorporated into the NSF of the embryo and yolk sac when the labeled substrate is injected into the yolk as early as the sixth day of incubation.

The results indicate that the yolk sac is a site of squalene and cholesterol biosynthesis. It is not known whether this synthesis occurs in the tissue itself or in the blood vessels associated with the yolk sac. It was observed that the yolk sac is capable of synthesizing a high level of labeled squalene. It is suggested that this tissue may be a promising site for a study of the formation of squalene from farnesyl pyrophosphate (10).

Labeled squalene-like and cholesterol fractions were identified in the NSF obtained from the embryos of incubating eggs. These components may have been synthesized within the embryo or transferred from the yolk sac during the incubation.

The results indicate that the NSF synthesized from MVA-2-C^{14} and the unlabeled NSF from the yolk pool are transferred to the embryo during incubation. How-

TABLE 3. SUMMARY OF THE RADIOACTIVITY OBTAINED IN THE ALUMINA COLUMN CHROMATOGRAPHY OF THE NONSAPONIFIABLE FRACTION FROM THE POOLED EMBRYO AND FROM THE POOLED YOLK SAC-MEMBRANE ISOLATED ON THE TENTH DAY OF INCUBATION OR FOURTH DAY AFTER THE INJECTION OF MEVALONIC ACID-2-C^{14}

Solvent	Material Eluted	Yolk Sac-Membrane Peak	Total Radioactivity	Percentage of Total*	cpm / mg LB+† Sterol	E Peak	Total Radioactivity	Percentage of Total*	cpm / mg LB+† Sterol
			cpm				*cpm*		
10% benzene	Squalene	A	39,000	40.6		A	2,110	5.9	
100% benzene	Components less polar	B	7,470	7.8		B	250	0.7	
20% diethyl ether	than cholesterol	C_1	2,880	3.0	945	C_1	195	0.5	
		C_2	5,860	6.1	20,700	C_2	110	0.3	
100% chloroform	Sterol, largely cholesterol	D_1	35,440	36.9	465	D_1	23,455	65.1	433
						D_2	7,685	21.3	418
95% ethanol	Components more polar	E_1	5,440	5.7	6,100	E_1	730	2.0	484
	than cholesterol					E_2	1,530	4.3	512

* The percentage of the counts present in each peak based on the total activity recovered from the column.
† The activity per milligram of Liebermann-Burchardt positive sterol.

ever, the synthesis of a NSF within the embryo is not excluded. Although the total activity in both the yolk sac and embryo increased throughout the incubation, the specific activity decreased, indicating transfer of unlabeled sterol from the yolk. An increase in the rate of synthesis of the labeled NSF, as indicated by the rapid increase in total activity, was seen in both the yolk sac and embryo in the last third of the incubation. The increased rate was observed in the yolk sac prior to that seen in the embryo. The cause of this sudden increase is unknown.

A labeled NSF was isolated from the liver, absorbed yolk sac, and carcass of chicks hatched from injected eggs. The brain was found to contain about 3% of the recovered activity. Recently, Budowski *et al.* (11) found a high specific activity in the NSF isolated from the brain tissue of chicks hatched from eggs obtained from laying hens that had received injections of glyceryl-1,3-C^{14} tripalmitate-1-C^{14}. The observations indicate that a "brain barrier" may not exist in the developing chick embryo.

It should be noted that, although the yolk sac and the embryo are capable of synthesizing a NSF, the utilization of this system during incubation may be slight since the yolk has such a high reserve of cholesterol in its lipid pool (12). Tsuji *et al.* (4) have shown that the total cholesterol of the whole egg, (i.e., yolk and embryo) does not increase greatly during incubation. Their data indicate that the total cholesterol of the egg before incubation was 261 mg. Although the total cholesterol of the yolk and embryo on the 21st day was 298 mg, the average value throughout the incubation was 260 mg.

The enzyme systems that convert mevalonic acid to cholesterol are functioning in the yolk sac and in the embryo from the sixth day of incubation. However, it appears that the embryo obtains its cholesterol primarily from the yolk stores.

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